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The ceaseless significance of lactic acid bacteria

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Abstract

The upcoming concern for the healthier lifestyle demands the first most obvious thing that is the healthy food intake. Apart from the enormous role of lactic acid bacteria in promoting health benefits of the food by their direct involvement in food fermentations, here we will be discussing the general characteristics and their importance along with the recent tools and techniques by quoting *Lactococcus lactis* as a model, leading to their increased utilization in industries for variety of purposes.

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Introduction

The term "lactic acid bacteria" comprises a diverse group of bacteria that are intricately linked to humans and animals. These microbes occur naturally in different environments, such as, the gastrointestinal, oral and respiratory tracts and are also found in food products such as milk, meat, plant products and wine (Van Reenen & Dicks, 2011). Each individual has a specific microbiome playing direct role for maintaining health of the host. Several species of gut microbes have been identified by various techniques and the composition of this metabolically active microbiota is linked to many disease states (Gueimonde & Salminen 2004).

LAB are commonly rod or cocci shaped Gram-positive, low-GC, non-motile, non-sporulating and non-respiring microbes which include the genera: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weiss Ella* (Michael E. Stiles & Holzapfel, 1997) (Makarova *et al.*, 2006). The classification into different genera is based upon the morphology, carbon metabolism, growth in the range of temperatures, acid, alkaline and salt stress tolerance (Khalid, 2011). Apart from the diverse characteristics, LAB are also related metabolically and physiologically. PCR based methods targeting 16S rRNA was used few decades back to determine the relatedness of LAB associated with food products leading to changes in their taxonomic classification. LAB are also classified on the basis of GC content lower and upper than 50% (Michael E. Stiles & Holzapfel, 1997) (Calo-mata, Arlindo, Boehme, & Barros-velazquez, 2008).

LAB are involved in food fermentations and used as starter cultures for the production of fermented food products where they inhibits the growth of pathogenic microbes such as *Clostridium* and *Staphylococcus* by acidification of the environment due to production of large amount of lactic and thus lowering the pH of the food products. LAB also contribute flavor to the food by their metabolism and helps in preserving and improving nutritional qualities of the food (M E Stiles, 1996).

LAB ferment sugars via three different pathways resulting in homo-, hetero-, or mixed acid fermentation. Under anaerobic conditions homofermenters produce only lactic acid through Embden-Meyerhof-Parnas pathway (Thomas, Ellwood, &Longyear, 1979) (Smith, Hillier, Lees, & Jago, 1975). This high metabolic rate of lactic acid production is due to higher activity of lactate dehydrogenate leading to the regeneration of NAD+. Due to this focused phenomenon it has become easy to redirect the metabolic fluxes towards the production of other metabolites as well (Jeroen Hugenholtz and Michiel Kleerebezem, 1999). Heterofermenters produce equimolar amounts of lactic acid, carbon dioxide and ethanol or acetate through phosphoketolase pathway, redox potential of the system determines the ratio of ethanol and acetate produced (Axelsson, 1998) (Garvie, 1980) (Kandler, 1983).

Mixed acids are produced by homofermenters such as *Lactococcus lactis* during glucose limitation (Fordyce, Crow, & Thomas, 1984), during growth on other sugars (Karin Hofvendahl & Hahn-Hägerdal, 1997)(Åkerberg C, Hofvendahl K, Zacchi G, 1998) (Qian, Stanley, Hahn-Hagerdal, & Radstrom, 1994) (Garrigues *et al.*, 1997) (Thomas, Turner, & Crow, 1980), or at increased pH and decreased temperature (K. Hofvendahl, Van Niel, & Hahn-Hägerdal, 1999). In general the increased carbon flux through the pathways results in homolactic mode while the limited carbon flux results in accumulation of mixed acid products (Garrigues *et al.*, 1997). The same homofermentive pathway is utilized during the mixed acid fermentation; only the metabolism through the pyruvate node differs, resulting in formation of ethanol, acetate and formate in addition to lactic acid (Karin Hofvendahl, 2000).

Characteristics of lactic acid bacteria

Genomics

The size of the genome of typical LAB ranges from 1.7 Mbp to 3.4 Mbp with a GC content of 35%-50%. Thousands of LAB strains have been completely sequenced with the advancement of next generation sequencing technology.

LAB shows a reductive genomic evolution, which means the reduction in genome size, most likely due to adaptation in nutrient rich environments, from plant to milk, which has been shown in *L. lactis* isolated from plant to adaptive evolution in milk (Douillard & de Vos, 2014) (Bachmann, Starrenburg, Molenaar, Kleerebezem, & Vlieg, 2012). The reduction in genome size is due to the prevalent presence of pseudo genes (genes that have lost their function during the course of evolution), which is most often seen in LAB than in other bacteria (Makarova *et al.*, 2006) (Schroeter & Klaenhammer, 2009). Thus the loss of genes during the course of evolution is complemented by the enhancement of genes required for the uptake of amino acids, sugars, peptides, etc. to take up the nutrients from the environment instead of synthesizing them denovo (Schroeter & Klaenhammer, 2009) (Douillard & de Vos, 2014). LAB genomes also contained transposons and many LAB harbor plasmids required for the growth in specific environments and carry genes required for growth in milk (Siezen *et al.*, 2005). So the plasmids and other mobile genetic elements have shaped the evolution of LAB by facilitating horizontal gene transfer (Douillard & de Vos, 2014)

Metabolism

LAB lacks the functional electron transport chain and hence they rely on substrate level phosphorylation for ATP synthesis. Substrate uptake is mediated by the phosphoenolpyruvate (PEP)- dependent phosphotransferase system, which is also the main system for carbon catabolite repression, and a major regulator of carbohydrate metabolism in bacteria. Sugar is also transported by sugar- specific permeases alternatively (Postma, Lengeler, & Jacobson, 1993) (Carr, Chill, & Maida, 2002). The extra cellular substrate is utilized by the two modes of fermentation pathways:

1. *Homofermentation pathway*

This employs the typical Embden-Meyerhof-Parnas (EMP) pathway that involves the oxidation of one mole of glucose into two moles of pyruvate concomitant with two moles of ATP generation. Two moles of pyruvate are then reduced to two moles of lactate by enzyme lactate dehydrogenase, thus regenerating the NAD^+. Mannose and galactose can also be utilized through PTS or specific permeases that can enter into glycolsis by conversion into glucose-6-phosphate through the Leloir pathway or tagatose-6-phosphate pathway (Salminen & Wright, 2011). This pathway leads to the conversion of over 95% of sugar into lactic acid and hence called the homofermentation pathway (Gaspar, Carvalho, Vinga, Santos, & Rute, 2013).

2. *Mixed acid fermentation pathway*

In this condition the pyruvate formed in the EMP pathway is not totally converted into lactate, instead pyruvate is processed by pyruvate-formate lyase or pyruvate dehydrogenase to produce acetyl-CoA that is further converted into acetate and ethanol (Thomas *et al.*, 1979) (Kandler, 1983). The significant amounts of lactate along with acetate and ethanol are called mixed acid products. Pyruvate is also converted into α-acetolactate leading to the formation of diacetyl, acetoin and 2,3-butanediol that are important flavor compounds in dairy industry (Salminen & Wright, 2011).

3. *Heterofermentation pathway*

This involves the entry of either pentose or glucose-6-phospahte directly into pentose phosphate pathway instead of EMP pathway. A reaction catalyzed by phosphoketolase leads to the formation of glyceraldehyde-3-phosphate that enters glycolysis and acetyl phosphate that further forms acetate and ethanol (Khalid, 2011) (Salminen & Wright, 2011) (Kandler, 1983).

Proteolytic system

The ability of LAB to adapt in nutritional rich environment is very important characteristic as they show reduction in genome size; so the loss of ability to synthesize nutrients is complemented by gain in ability to utilize nutrients from the environment (Schroeter & Klaenhammer, 2009) (Douillard & de Vos, 2014). This is possible due to the proteolytic ability of LAB, particularly those adapted to dairy environment, as they encode cell surface proteinases to break the protein into range of peptides that can be further taken up by specific transport systems (Kunji, Mierau, Hagting, Poolman, & Konings, 1996).

Stress tolerance

Tolerance to different type of stresses is an important characteristic that distinguishes different species of LAB. One of the examples of self-imposed stress is lactic acid stress that comes through the accumulation of lactic acid, a major end product of sugar fermentation. LAB are relatively acid tolerant but ultimately the cell physiology is affected by the acid stress (Even *et al.*, 2002). Food related LAB encounter drastic temperature fluctuations and they counteract the effects of heat stress by the induction of various heat shock proteins (HSPs) (Craig, 1985). LAB are less sensitive to osmotic stress commonly encountered in food industry as compared to several pathogenic microorganisms (Sleator & Hill, 2001). LAB are usually microaerophilic lacking a functional electron transport chain and catalases, however some of them are aero tolerant.

They have been shown to undergo respiration if external heme and/or menaquinones are supplied(Pedersen, Gaudu, Lechardeur, Petit, & Gruss, 2012). The cell envelope acts as first line of defense against any environmental changes, modification in the chemical composition of both cell wall and the membrane induced by stress have been shown in increasing cell survival (Bush, 2012) (Papadimitriou *et al.*, 2016). Actually LAB inhabits in different stress environments that are mostly nutritious to overcome their auxotrophies. Although none of the LAB has been categorized initially as an extremophile, but there are reports showing several species or strains that can tolerate or even grow in harsh environments (Mills, Stanton, Fitzgerald, & Ross, 2011) (Sheh & Fox, 2013) (Kleynmans, Heinzl, & Hammes, 1989) (Lo *et al.*, 2004).

Importance of lactic acid bacteria

Probiotics

Probiotics are defined as "living microbes, which upon intake in certain numbers exert health benefits beyond inherent basic nutrition". LAB strains including *Lactobacillus, Enterococcus and Bifidobacterium* species have also been found to exert probiotics benefits when they are consumed as food components or as food supplements (Guarner & Schaafsma, 1998).

They are the natural residents of the human gut and along with the other bacterial species form the intestinal 'microflora'. They can withstand the low pH of the stomach and colonize the large intestine where they secrete antimicrobial compounds and antioxidants that inhibit the growth of pathogens and scavenge free radicals. Also they reside in the intestinal surface thus preventing other microbes entering the body (Ljungh & Wadström, 2001).

Starter cultures

LAB play a major role in food fermentation where they are the primary constituent of industrial starter cultures involved in the production of a variety of dairy products. The type of starter cultures used for the fermentation determines the quality of the fermented products such as aroma, shelf life, and preservation. Starter cultures of different LAB species contributes differently to final flavor and texture of the food products (Smit, Smit, & Engels, 2005). Some LAB species such as *Lactococcus*, *Lactobacillus*, *Pediococcus* and *Enterococcus* can be used in the preservation of fermented meats, fish, vegetables, soy sauce, wine etc. by the combined effect of the bacteriocin and lactic acid production that inhibits the growth of pathogenic bacteria and help LAB to dominate the microflora of the food products (M E Stiles, 1996) (Klaenhammer, 1993).

Cell factories

The long traditional use of LAB in food fermentation and by humans has provided LAB a generally recognized as safe (GRAS) status by the US Food and Drug Administration Agency (Gaspar *et al.*, 2013). LAB has a relatively simple carbon metabolism that makes them important targets for metabolic engineering for the production of food ingredients, nutraceuticals and also non-food related commodity chemicals. There are various examples from each category such as alanine which is a natural sweetener and used as food additive, Nice system has been used to overexpress the alanine dehydrogenase from *Bacillus sphaericus* in LDH deficient strain of *L. lactis* for homoalanine production (Hols *et al.*, 1999). Diacetyl is an important flavor compound contributing the buttery aroma of dairy products is a side product of LAB metabolism.

Different metabolic engineering strategies have been used for diacetyl production from glucose or lactose instead of citrate that follows the natural means of diacetyl synthesis by LAB through citrate utilization (Gosalbes, Esteban, Galan, & Perez-Martinez, 2000) (Kleerebezemab, Hols, & Hugenholtz, 2000). Also, acetaldehyde is an important aroma compound like diacetyl specifically in yogurt, *L. lactis* over expressing pyruvate decarboxylase from *Z. mobilis* along with native NADH oxidase leads to high level acetaldehyde production (Bongers, Hoefnagel, & Kleerebezem, 2005). Various LAB has been exploited for production of high value metabolites such as polyols, vitamins and exopolysaccharides.

Polyols or sugar alcohols are common sugar substitutes in food products; xylitol, mannitol and sorbitol are the most widely used polyols whose production has very well shown in various LAB strains (Monedero, Pérez-Martínez, & Yebra, 2010). LAB are attractive targets for vitamin overproduction as they have the ability to synthesize B vitamins (Sybesma, Burgess, Starrenburg, Van Sinderen, & Hugenholtz, 2004) (Santos, Wegkamp, De Vos, Smid, & Hugenholtz, 2008). Increased EPS production through metabolic engineering in *L. lactis* has also been achieved using NICE system (Looijesteijn, Boels, Kleerebezem, & Hugenholtz, 1999). LAB has been used in industries for large-scale production of lactic acid due to their ability to convert over 90% of sugar into lactic acid.

Lactic acid is a raw material for pharmaceutical industries and biodegradable plastic industries, LAB strains has been tailored to produce optically pure L-lactic acid through fermentative processes (Kylä-Nikkilä, Hujanen, Leisola, & Palva, 2000). *L. lactis* has also been engineered for ethanol production by introducing genes from *Zymomonas mobilis* (Christian Solem, Dehli, & Jensen, 2013). 2,3-butanediol along with mannitol has been produced in *L. lactis* by cofactor engineering (Gaspar, Neves, Gasson, Shearman, & Santos, 2011). Also, LAB, mainly *Lactococcus lactis*, have been developed into cell factories for the production of hydrolytic enzymes and therapeutic proteins (Vos & Hugenholtz, 2004) (Cammarota *et al*., 2000) (Steidler *et al*., 2000).

Various LAB have also been exploited for the production of recombinant proteins due to the availability of food-grade controlled gene expression systems, of which the nisin controlled gene expression system has gained the much popularity (Mierau & Kleerebezem, 2005).

Lactococcus lactis at a glance

Lactococcus lactis belongs to the group of lactic acid bacterium under the family Streptococcaceae. It is gram-positive cocci, mesophilic growing optimally at 30 degress and pH=7. It can be isolated from plants or dairy environments (Rademaker *et al*., 2007). *L. lactis* has two subspecies namely subsp. *lactis* and subsp. *cremoris*.

The common examples from both the subspecies are the most widely used laboratory strains IL1403 and MG1363. The IL1403 was derived from the *L. lactis* subsp. Lactis biovar diacety lactis *CNRZ157* by curing the citrate plasmid while MG1363 is a plasmid-free derivative of the dairy strain NCDO712 (Chopin, 1984) (Gasson, 1983). Among all LAB, *L. lactis* is one of the most widely studied organism of this group, due to its tremendous industrial importance.

It is used as starter in the dairy industries for the synthesis of fermented food products (Kelly, Ward, & Leahy, 2010). Lactose is a major carbon source found while growth of these bacteria in milk, however they have the ability to consume various mono and disaccharides as substrates. Lactic acid is the primary fermentation product produced during anaerobic conditions, known as homolactic fermentation. It also undergoes mixed acid fermentation during micro aerobic condition producing significant amounts of formate, acetate and ethanol. Also metabolism of LAB is very important for contributing the final product properties like flavor, texture and shelf life (Kleerebezemab *et al*., 2000). *L. lactis* has been engineered to become a cell factory for the production of wide variety of chemicals including recombinant proteins, therapeutic proteins, vaccine antigens, flavor ingredients and nutraceuticals etc. (Morello, Llull, Miraglio, Langella, & Poquet, 2008) (Bahey-el-din, Gahan, & Griffin, 2010) (Vos & Hugenholtz, 2004) (Vuyst, 2004).

Tools for studying Lactococcus lactis

A large number of tools have been developed to manipulate cells at the molecular level. With the advancement of different omics-techniques one can study the biology of the cell at the systems level. Apart from the tools designed for DNA manipulation, two important tools has been specifically developed for modulating gene expression which are first established in *L. lactis* and later applied to other LAB. One is the construction of synthetic promoter libraries and another is the inducible gene expression system.

1. Controlled gene expression systems

NICE system is the most commonly known controlled gene expression system used for the lactic acid bacteria (Mierau & Kleerebezem, 2005)(Kuipers, Ruyter, Kleerebezem, & Vos, 1998). There are various advantages of using nisin as an inducer for overexpression of recombinant proteins. The nisin is considered to be safe to use that makes the system food grade and it is highly sensitive, so very small amount of it is required for induction (0.1- 5 ng/ ml) that does not inhibits the growth of other microbes in a starter culture during fermentation (Mierau & Kleerebezem, 2005) (Kuipers, de Ruyter, Kleerebezem, & de Vos, 1997). Also the expression level is linear with the amount of inducer used in a dynamic range that can be more than 1000 folds (Vos, 1995) (Willem, 1996).

Similar systems using another bacteriocin sakacin as inducer was developed for other LAB as well (Sorvig, Mathiesen, Naterstad, Eijsink, & Axelsson, 2005) (Nguyen *et al.*, 2011). Also attempts have been made to develop zinc controlled gene expression systems in *L. lactis* (Llull & Poquet, 2004).

2. *Synthetic promoter libraries*

A synthetic promoter library (SPL) consists of a library of promoters with the fixed consensus sequences and randomized spacers in between (Hammer, Mijakovic, & Jensen, 2006) (Dehli, Solem, & Jensen, 2012) (Mijakovic, Petranovic, & Jensen, 2005). SPL has been shown in modulating gene expression in a dynamic range of up to thousand folds (Peter Ruhdal Jensen & Hammer, 1998) (P R Jensen & Hammer, 1998).

SPL results in continuous range of activity in comparison to traditional approaches that involves either knockout of a gene of interest or its overexpression by a strong promoter. This approach has been used to study glycolytic flux control in *L. lactis* (Koebmann, Solem, & Jensen, 2006) (C Solem, Koebmann, & Jensen, 2008) (Christian Solem, Petranovic, Koebmann, Mijakovic, & Jensen, 2010). This method has also been used in other LAB such as *L. plantarum* (Rud, Jensen, Naterstad, & Axelsson, 2006).

Other genetic tools

Apart from inducible gene expression systems and SPL, some basic genetic tools are also important for studying LAB. Numerous plasmids have been generated for creating indels in chromosomal DNA of *L. lactis* by homologous recombination. One example is pINT1 and another is pGhost system, both are derived from pWV01 plasmid, one is non-replicating and another has thermo-sensitive replication (Otto, Vos, & Gavrieli, 1982) (Maguin, Duwat, Hege, & Ehrlich, 1992)(Biswas, Gruss, Ehrlich, & Maguin, 1993). After that the pORI series was created to make use of both the above systems (Leenhouts, Venema, & Kok, 1998) (Law *et al.*, 1995).

Later the pCS1966 was developed which was derived from pBluescript to manipulate the chromosome (Le Bourgeois, Lautier, Mata, & Ritzenthaler, 1992). It involves the selection of integration at non-permissive temperature and counter selection at permissive temperature confirming the excision of plasmid. Erythromycin is used as selection marker for integration and 5-fluoroorotate; a toxic pyrimidine analogue is used as counter selection marker to confirm the excision of plasmid from the chromosome (Christian Solem, Defoor, Jensen, & Martinussen, 2008).

A derivative of pCS1966, pSEUDO plasmid, was also designed for integration into a pseudo locus (neutral region) in *L. lactis* (Pinto *et al.*, 2011). Transposes based approaches have also been designed for chromosomal integrations in other LAB.

Although site-specific integration systems originating from temperate bacteriophage naturally exist in many LAB such as *Lactobacillus* and *Streptococcus thermopiles* (Goh *et al.*, 2009) (Douglas & Klaenhammer, 2011).

There have always been advances in development of molecular biology tools for easing the process of chromosomal DNA manipulation. Recently ssDNA recombinering have become very popular for silencing the effect of targeted locus (Pijkeren & Britton, 2012). Along with the existing tools for recombine ring CRISPR/Cas9 based genome editing has become much popular (Jiang, Bikard, Cox, Zhang, & Marraffini, 2013) (Sander & Joung, 2014) (Gomaa *et al.*, 2014) (Selle, Klaenhammer, & Barrangou, 2015) (Selle *et al.*, 2015). The CRISPRs (clustered regularly interspaced short palindromic repeats)/Cas9 employs the RNA-guided DNA editing technology to introduce double stranded breaks into genomes leading to specific, markerless insertions/deletions or replacement of targeted locus (Huang, Zheng, Jiang, & Hu, 2015).

The gram-positive bacteria are infamous for being difficult to engineer, however using CRISPR/Cas9 assisted homologous recombination it has been possible to do clean gene deletions in *Clostridium beijerinckii* NCIMB 8052 (Wang *et al.*, 2015). A combined approach of single-stranded DNA (ssDNA) recombinering along with the CRISPR–Cas9 have been shown in LAB *Lactobacillus reuteri* by Jee-Hwan Oh and Jan Peter van Pijkeren (Oh & van Pijkeren, 2014). In general CRISPR/Cas9 mediated genome editing has potential to modify the genome of LAB and other gram-positive bacteria with reduced off target effects (Oh & van Pijkeren, 2014).

Conclusion

The increasing applications of lactic acid bacteria demands for the development of more tools that can be extended to other LAB along with *Lactococcus lactis*. With the recent advancement of genome level manipulation tools one can exploit the microbe's machinery to stably produce their product of interest.

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